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Full Length Research Paper

Studies on improved *Agrobacterium*-mediated transformation in two *indica* rice (*Oryza sativa* L.)

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Agrobacterium tumefaciens strain EHA 105 carrying binary vector pCAMBIA 1301 was used for transformation in two economically important highly recalcitrant indica rice cultivars HKR-46 and HKR- 126. High concentrations of acetosyringone in the Agrobacterium culture and co-cultivation medium proved to be indispensable for successful transformation. Embryogenic scutellar calli were used for transformation studies. Binary vector pCAMBIA 1301 have been proved efficient for transformation. The percent transient GUS expression found to be higher in cultivar HKR-126 (44.4%) as compared to HKR- 46 (28.9%). The percent recovery of hygromycin resistant calli after 4-6 weeks on selection medium was maximum in HKR-126 (52.6%).

Key words: *Agrobacterium*, *indica* rice, transformation, acetosyringone.

INTRODUCTION

Rice consumers are increasing at the rate of 1.8 per cent every year. But the rate of growth in rice production has slowed down. It is estimated that rice production has to increase by 50 per cent by 2025 (Khush and Virk, 2000). This will require acceleration in rice production. Solving this problem will entail development of rice varieties, which have higher yields, excellent grain quality, and resistance to biotic and abiotic stresses. To expedite genetic improvement in rice, genetic engineering can be used as a powerful and novel tool to complement the traditional methods of plant improvement. It permits access to an unlimited gene pool through the transfer of desirable genes (Hiei et al., 1997).

Japonica and *indica* are the two major subspecies of rice grown in different region of the world. *Indica* rice (long grain tropical rice) alone accounts for approximately 80 per cent of the cultivated rice. *Indica* rice is sensitive to several biotic (yellow stem borer, stripped stem borer, leaf folders susceptible to blight and blast) and a biotic (drought and salinity) stresses and is a poor yielder. Cowpea trypsin inhibitor gene has been reported to be effective against yellow stem borer and stripped stem borer in transgenic *japonica* rice (Xu et al., 1996). Bacterial blight resistant gene Xa21 has been introduced through transformation into a high yielding *indica* variety IR72 (Tu et al., 1998) that showed excellent field performance (Tu et al., 2000). Incorporation of capsid protein genes has provided protection against infection from virus in rice (Hayakawa et al., 1992; Sivamani et al., 1999). Cloned genes and transgenic plants have now become a standard tool in plant stress biotechnology. An array of stress regulated

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Abbreviations. 2,4-D: 2,4-Dichlorophenoxy acetic acid, X-Gluc: 5-bromo, 4-chloro, 3-indotyl glucuronic acid, CH: Casein hydrolysate, Vir: Virulence.

Table 1. Media used for rice transformation experiment.

Medium	Culture media constituents
Callus induction medium	MS basal, 2,4-D 2.5 mg/l, Proline 500 mg/l, CH 500 mg/l, 2.5 mg/l gelrite, Sucrose 30g/l and pH – 5.8
Liquid co-cultivation medium	Same as callus induction medium + sucrose 68.5 g/l, Glucose 35 g/l and pH - 5.2 (no gelrite)
Co-cultivation Medium-I	Same as callus induction medium + Sucrose 30 g/l, Glucose 10 g/l, acetosyringone100 µM and gelrite 2.5 g/l
Co-cultivation medium-II	Same as co-cultivation medium-I but acetosyringone 200 µM
Co-cultivation medium-III	Same as co-cultivation medium-I but acetosyringone 400 µM
Co-cultivation medium-III	Same as co-cultivation medium-I but acetosyringone 400 µM
Co-cultivation medium- IV	Same as co-cultivation medium-I but acetosyringone 500 µM
Selection medium	Same as co-cultivation medium-I + hygromycin 50 mg/l, Carbenicillin 250 mg/l, pH – 5.8 (no acetosyringone)

genes have been isolated (Bartels and Nelson, 1994; Bajaj et al., 1999). Functions of some of these genes are close to be identified and their role in stress physiology is being determined.

Agrobacterium-mediated transformation has been reported in selected *indica* rice cultivars (Khanna and Raina et al., 1999; Rashid et al., 1996) and *japonica* rice (Hiei et al., 1997; Ilag et al., 2000; Chern et al., 2001). Before targeting a particular rice cultivar to in vitro genetic manipulation, we need to have an efficient system for regeneration of green fertile plants from explants tissues. Our previous report has described efficient regeneration system for these two cultivars; in addition these two *Indica* rice cultivars have proven to be a difficult material for in vitro culture (Saharan et al., 2004). In fact, recalcitrant nature of this subspecies has been a major limiting factor for successful transfer of available useful genes. Till date, most of the transformation studies were conducted on *Japonica* and some of *indica* rice cultivars which posses the quality features required of exported rice. Due to these factors more attention is required to improve some existing economical important local cultivars. In the present study, the condition was optimized for *Agrobacterium*-mediated genetic transformation in two recalcitrant *indica* rice HKR-46 and HKR-126.

MATERIALS AND METHODS

Seeds of two elite dwarf and high yielding rice cultivars HKR-46 and HKR-126 were collected from CCS Haryana Agricultural University, Rice Research Station, Kaul (Haryana), India. HKR-46 is a short duration cultivar (135 days) whereas HKR-126 is a medium duration cultivar (140 days). These cultivars essentially belong to varietal group-1 based on the isoenzyme polymorphism (Glaszmann, 1987). For transformation experiments bacterial strain (*Agrobacterium tumefaciens*) was provided by CAMBIA, Australia.

Media composition

Different media were prepared using the modified MS medium (Murashige and Skoog, 1962). The chemical composition of different media for *Agrobacterium*-mediated transformation are described in Table 1. All the chemicals were purchased from Sigma Company.

Callus induction

Dehusked seeds were washed in 70% ethanol for 1 min and then rinsed with sterilized water to remove traces of ethanol. Sterilization of seeds was carried out on shaker using a solution of sodium hypochlorite (with 2% active chlorine) and Tween-20. After 40 min, the solution was removed and seeds were thoroughly washed 5-6 times with sterilized water. Sterilized seeds of the two cultivars transferred into petri dishes containing callus induction medium (Table 1). After 3 weeks of incubation under dark 25±1°C, calli initiated from scutella were sub cultured on fresh callus induction medium.

Transformation procedure

Agrobacterium Strain EHA 105 containing pCAMBIA 1301 was grown on AB minimal media (Ilag et al., 1999) having 50 mg/l hygromycin and 50 mg/l kanamycin for 2-3 days at 28±1°C in the dark. Agrobacgerium was scrapped from AB minimal media plates and resuspended in liquid co-cultivation medium supplemented with 100 µM acetosyringone on rotary shaker (150 rpm) for 30 min. Optical density for the bacterial suspension was measured at OD600. The bacterial suspension having optical density 1.5 to 2 was used. Three weeks old calli were excised from mature seeds from both the cultivars and sub-cultured on callus induction medium for 4-5 days. Approximately 1-2 mm size embryogenic calli were immersed in *Agrobacterium* suspension for 30 min. Agro-infected calli were blotted dry on sterile Whatman No. 1 filter paper and transferred on to the co-cultivation medium under dark at 25±1°C for three days.

Gus assay

Histochemical GUS assay was carried out after three days of co-cultivation using GUS assay consisting of sodium phosphate buffer and X-Gluc as the substrate. Callus pieces were dipped in GUS assay solution in ELISA plates and incubated at 37°C for 24 h.

Selection of transformed calli

After co-cultivation, *Agrobacterium* infected calli were washed with sterilized distilled water containing carbenicillin 250 mg/l in order to kill the *Agrobacterium*. These washed calli were transferred on to the selection medium containing hygromycin (50 mg/l) and carbenicillin (250 mg/l). After three weeks, calli were sub cultured on to the fresh selection medium. The cultures were incubated at 25±1°C in dark. After 4-6 weeks, data was recorded on the number of calli showing growth on selection medium.

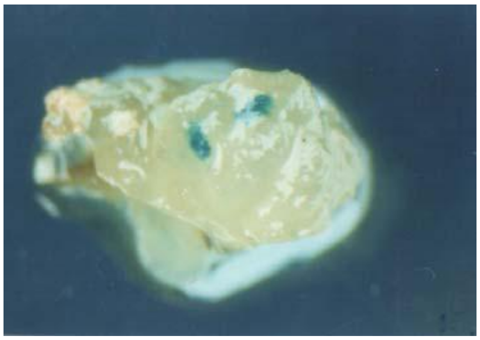

Figure 1A. Transient *GUS* expression - Indicating as blue color patches on callus.

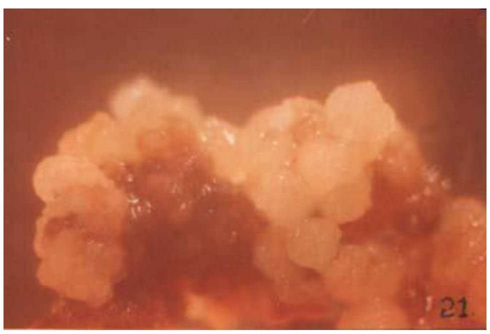

Figure 1B. Embryogenic hygromycin resistant calli on selection medium after 4-6 weeks - Globular callus showed sustained proliferation at one or more regions.

RESULTS AND DISCUSSION

Calli formation was invariably developed from the scutellar region of the seeds and was visible within 7-10 days. Freshly subcultured embryogenic calli were subjected to transformation.

Transient GUS- expression: Histochemical GUS assay was carried out to assess the expression of GUS gene in the calli as described by Rueb et al. (1989). Blue patches indicated Gus activity, confirming GUS expression in co-cultivated callus tissues (Figure 1A). Maximum number of calli showing blue color with maximum frequency of blue patches were found on those calli co-cultivated with co-cultivation media-III. It was evident that increase in concentration of acetosyringone enhance the GUS activity (Figure 2). The percent transient GUS expression in HKR-46 and cutivar HKR-126 were 28.9 and 44.4%, respectively, on co-cultivation media-III (Table 2).

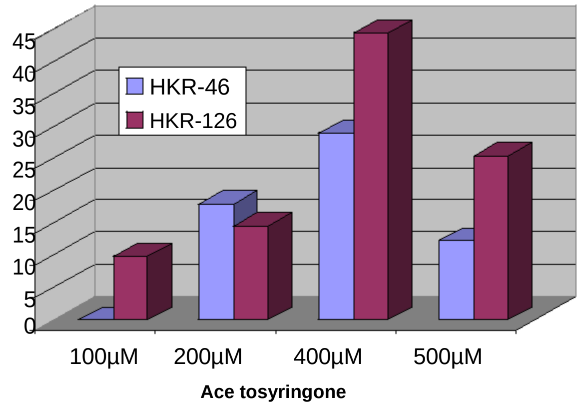

Figure 2. Effect of acetosyringone on *GUS*-activity in mature seed derived calli of HKR-46 and HKR-126 cultivars.

Table 2. Transformation efficiency in both cultivars HKR-46 and HKR-126.

Cultivar	**% Transient *GUS*-expression[x]**	**% Recovery of hygromycin resistant calli [y]**
HKR-46	28.9 ± 1.8	35.0 ± 1.0
HKR-126	44.4 ± 1.0	52.6 ± 2.0

[x]Observed after 3 days on co-cultivation media-III
[y]Observed after 4-6 weeks on selection media
The experiment was repeated thrice, each experiment consists of 20 calli with 3 replication.

Selection of transformed cells/calli: The calli after co-cultivation was transferred to the selection medium for 2 to 3 cycles of selection (15 days each) containing callus induction medium with 50 mg/l hygromycin and 250 mg/l carbenicillin. After 2-3 weeks on selection medium, calli showed sustained proliferation at one or more regions. Continuous selection on hygromycin containing medium resulted in the appearance of proliferating apparently resistant embryogenic calli (Figure 1b).

The gene encoding β -D-glucuronidase (GUS) has been the most widely used reporter gene for the analysis of plant gene expression in plant transformation systems described by Jefferson et al. (1997). The GUS expression system has been further improved by cloning an intron within the GUS gene region, which completely inhibits its expression in *Agrobacterium*, thus allowing the precise visualization of transformation events at early stages after the co-cultivation. Several critical factors including *Agrobacterium* strains and vectors, use of acetosyringone for the induction of vir genes, competence of the rice genotype, co-cultivation period and conditions and tissue culture media have been reported to affect transient GUS expression and transformation efficiency in rice (Hiei et al., 1997; Khanna and Raina, 1999).

In these two cultivars acetosyringone played crucial role for improvement and efficient transformation. Vector pCAMBIA 1301 used in the present study, have been proven ideal for rice transformation (Ilag et al., 2000; Chern et al., 2001). We have used a modified MS medium containing acetosyringone, 2,4-D and casein hydrolysate with acidic pH of 5.2 for co-cultivation of embryogenic rice calli with *Agrobacterium* strain at 28±1° C. The addition of acetosyringone in co-cultivation medium has been reported to induce vir genes, extend host range of some *Agrobacterium* strains, and found essential for rice transformation (Hiei et al., 1994; Godwin et al, 1991). The expression of GUS was detected after 3 days on co-cultivation with *Agrobacterium* (transient assay). The level of transient GUS expression after co-cultivation with *Agrobacterium* in the two rice cultivars varied with genotype. Percent transient GUS expression observed in this study were comparable to earlier reports in *indica* rice cultivars (Rashid et al., 1996).

Efficient plant selection during transformation requires a substantial level of expression of the selectable marker gene (Wang et al., 1997). In the present study, calli co-cultivated with EHA 105 strain containing pCAMBIA 1301 were allowed to grow and subjected to two cycles of selection of 15 days each in the callus induction medium containing 50 mg/l hygromycin and 250 mg/l carbenicillin. Continuous selection (4 to 6 weeks) on hygromycin medium resulted in the selective proliferation of resistant calli. Hygromycin allowed clear distinction between transformed and non-transformed calli. The hygromycin phosphors transferase (HPT) gene has been used as an efficient marker gene for selection of transformed tissues (Rashid et al., 1996). In the present study, the hygromycin resistant calli 35% in HKR-46 and 52.6% in HKR-126 (Table 2). Recovery of hygromycin resistant calli were similar to earlier reports in *Japonica* and in *indica* rice cultivar (Rashid et al., 1996; Khanna and Raina, 1999). The differences in callus induction, transient GUS assay, recovery of hygromycin resistant calli and plant regeneration varied in the two *indica* rice varieties. HKR-46 has been found to be most recalcitrant to the induction of embryogenic calli and showed recovery of GUS calli and hygromycin resistant calli at lower percent. HKR-46 and HKR-126 are high yielding cultivars, which are sensitive to biotic and abiotic stresses. The developed transformation protocol will be of immense value to transform *indica* rice cultivars in general and these two cultivars in particular. Efforts are being made to regenerate transgenic plants and carry out molecular analysis.

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